

ROLE OF HEREGULIN IN BREAST CANCER ANGIOGENESIS

**Javier A. Menendez, I. Mehmi, E. Atlas,
M-S. Tsai, and R. Lupu**

Lawrence Berkeley National Laboratory,
One Cyclotron Road, Berkeley, CA

eatlas@lbl.gov

We recently established that CYR61, an angiogenic factor, is a downstream effector of Heregulin (HRG) action. Both HRG and CYR61 enhance tumor neovascularization, which may contribute to a more aggressive disease. Interestingly, conventional cytotoxic anticancer drugs have been discovered to have anti-angiogenic effects. However, exploiting chemotherapeutic drugs as anti-angiogenics could be compromised by the high concentrations of pro-angiogenic survival/growth factors present in the tumor microenvironment. We hypothesized that in addition to their role as pro-angiogenic factors, HRG and/or CYR61 can also modify chemotherapy effectiveness. To address this question, we first evaluated the impact of HRG expression in modulating breast cancer response to anticancer drugs. HRG-negative MCF-7 cells transfected with the full-length HRG cDNA (MCF-7/HRG) were assessed for cisplatin (CDDP), 5-Fluorouracil (5-FU), and paclitaxel (Taxol) sensitivity. MCF-7/HRG cells were significantly more resistant to the CDDP-induced effects on cell viability as compared to control cells. A weaker but significant increase in 5-FU resistance was observed in MCF-7/HRG cells. Also, MCF-7/HRG cells became more resistant to Taxol. Next, we studied whether ectopic expression of CYR61 alone, in HRG-negative MCF-7 cells, was able to modulate the sensitivity of breast cancer cells to chemotherapy. CYR61 transfectants and control cells were equisensitive to CDDP and 5-FU, however CYR61-transfected cells, were more resistant to Taxol, similarly to MCF-7/HRG cells. CYR61-induced Taxol resistance was reversed by the inhibition of phosphatidylinositol 3-kinase activity (PI3-kinase). In contrast to control cells, DNA from CYR61 transfectants treated with Taxol displayed no signs of the classical DNA laddering pattern of apoptotic death. In addition, MCF-7/CYR61 cells were unable to induce the pro-apoptotic p53 protein in response to Taxol-induced damage. Interestingly, functional blocking of the avb3 integrin, a CYR61 receptor, synergistically reversed the Taxol resistance. It is tempting to postulate that CYR61-induced activation of avb3-PI3 kinase-AKT pro-survival pathway is related to this phenotype. New therapeutic strategies based on HRG and/or Cyr61 may therefore, be developed.

ISOLATION OF SIGNALING MOLECULE INVOLVED IN ANGIOGENESIS MEDIATED BY BETA 5 INTEGRIN CYTOPLASMIC DOMAIN

Cardo-Vila, M., Arap, W., and Pasqualini, R.

M.D. Anderson Cancer Center

mcardovi@notes.mdacc.tmc.edu

Integrins that bind to vitronectin are highly expressed in multiple tumor types and in neovasculature, where they play an important regulatory role. Our objective was to define the molecules involved in α 5 β 3- and α 5 β 5-mediated signaling. We hypothesized that (i) different molecules associate with each of these integrins during malignant transformation and angiogenesis; and (ii) the assembly of specific molecules that associate with the β 3 or the β 5 cytoplasmic domain results in selective signaling. We have used a novel strategy to approach these hypotheses: panning of phage display peptide libraries on β 3 and β 5 cytoplasmic domains and determining the biological properties of the cytoplasmic domain-binding peptides. We successfully isolated distinct sequences that interact specifically with β 3 or with β 5 cytoplasmic domains. We used synthetic peptides corresponding to the sequence displayed by the phage to perform inhibitory studies and we found that they can inhibit the binding of corresponding phage in a dose-dependent manner. To determine the biological properties of peptides that bind to β 3 or β 5 integrin cytoplasmic domains, we used membrane-permeable forms of the peptides. We then evaluated the effects of selected cytoplasmic domain-binding peptides on β 3 or β 5 integrin-mediated signaling and subsequent cellular responses. We show that these membrane-permeable forms of the peptides are indeed internalized, can interfere with β 3 and β 5 post-ligand binding cellular events such as cell migration and proliferation. A peptide that binds specifically to the β 5 integrin cytoplasmic domain induced massive apoptosis. Cell killing was enhanced by stimulation of endothelial cells with VEGF, and abrogated in cells lacking α 5 β 5 isolated from β 5-null mice. Antibodies raised against two of our β 3 or β 5 cytoplasmic domain binding peptides recognize specific proteins, suggesting that the peptides isolated they may recognize key molecules involved in integrin signaling.

OVEREXPRESSION OF VEGF ENHANCES ESTROGEN-DEPENDENT AND -INDEPENDENT GROWTH OF MCF-7 BREAST TUMORS

Ping Guo,¹ Quan Fang,¹ Ivan Ding,² Bruce Fenton,²
Bo Hu,¹ and Shi-Yuan Cheng¹

¹University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213;

²Department of Radiation Oncology, University of Rochester, Rochester, NY 14642

chengs@msx.upmc.edu

Human breast cancers are dependent on estrogen or other estrogenic hormone for growth. Moreover, many estrogen-dependent breast tumors develop into more aggressive and malignant, estrogen-independent phenotype. Angiogenesis, a process of the formation of new blood vessels from preexisting vasculature, is important for the breast cancer growth. Vascular endothelial growth factor (VEGF) family, plays major roles in breast tumor angiogenesis. VEGF and its receptors are expressed at high levels in breast tumors. Modulation of VEGF functions regulated the angiogenicity and tumorigenicity of breast tumor cells in animals. VEGF regulates the functions of a similar set of estrogen (E₂)-modulated genes that contribute to breast cancer progression. In addition, E₂ directly regulates VEGF transcription expression by acting upon the estrogen response elements in the gene of VEGF. Early studies have demonstrated that factors induced by E₂ in E₂-dependent MCF-7 breast cancer cells could partially replace E₂ to promote tumor growth. Introduction of a human ras oncogene into these MCF-7 cells did not abrogate the E₂ dependency regarding for tumor growth in animals. Furthermore, E₂ dependent MCF-7 and T47D cells express VEGFR-1, VEGFR-2; and VEGF stimulates breast cancer cell mitogenesis. We therefore hypothesize that overexpression of VEGF in MCF-7 breast cancer cells might abolish E₂-dependency for breast tumor growth. To test our hypothesis, we constructed and characterized the MCF-7 breast cancer cell clones that overexpress two VEGF splicing isoforms, VEGF₁₂₁ and VEGF₁₆₅, as well as control LacZ gene. In vitro, the parental MCF-7 and MCF-7/LacZ cells express VEGF at low levels of 3.0 ng/ml/ 10⁶ cells in 48 hrs. The derived MCF-7 clone cells express VEGF₁₂₁ or VEGF₁₆₅ at high levels of 300 ng to 500 ng/ml/10⁶ cells. In cell culture, the MCF-7/VEGF expressing cells had increased proliferation rates than that of the parental MCF-7 cells and gained resistant to cell apoptosis induced by E₂-withdraw from the culture media. When these cells were implanted into mammary fat pads in mice that were inoculated with slow-release E₂ pellets, overexpression of VEGF₁₂₁ and VEGF₁₆₅ greatly enhanced E₂-dependent MCF-7 tumor growth. In 33 days, the parental MCF-7 or LacZ cells formed tumors in volumes of 250 mm³, whereas the MCF/VEGF expressing tumor established tumor in volumes of 1250 to 1500 mm³. Importantly, when the mice were no treated with E₂, no tumor were formed in mice that received the parental or MCF-7/LacZ cells. In contrast, in absence of E₂ treatment, the MCF-7/VEGF₁₂₁ or MCF7/VEGF₁₆₅ cells established tumors in mice with similar growth rate to that of the parental or MCF-7/LacZ tumors in mice exposed to E₂ treatments. Analyses of the angiogenesis of the various types of MCF-7 tumors demonstrated that no differences were found in vessel densities among all types of the MCF-7 tumors. However, in the parental and MCF-7/LacZ tumors that only formed in E₂ treated mice, most vessels were on the periphery of the tumors. In MCF-7/VEGF₁₂₁ or MCF-7/VEGF₁₆₅ expressing tumors established both in E₂ or non-E₂ treated mice, the neovessels extended into the centers of the tumors. The vessels in these types of tumors were highly perfused, whereas vasculature in other types of tumors was less accessible. Our data demonstrate that in addition to stimulating breast tumor angiogenesis, VEGF might mediate, at least in part, estrogen responsiveness in human breast tumor growth or VEGF might activate subsets of genes that render E₂-independent breast tumor formation in mice.

LYMPHANGIOGENESIS FROM POROUS ALGINATE-VEGF-C HYDROGELS IN VITRO

Mauricio A. Contreras, M.D., and Sumner Slavin, M.D.

Beth Israel Deaconess Medical Center,
Harvard Medical School

mcontrer@caregroup.harvard.edu

Introduction: The lymphatic vascular system develops from embryonic veins through a process referred to as Lymphangiogenesis. This event occurs in parallel with maturation of the blood vascular system, aberrant lymphangiogenesis contributes to interstitial protein accumulation, leading to a continual increase of osmotic pressure and thus fluid accumulation. Lymphedema therefore, ensues as a result of lymphatic blockage, trauma or dysfunction.

Objective: To promote Lymphangiogenesis by stimulating lymphatic endothelial cell (LEC) proliferation and migration using an Alginate-VEGF-C hydrogel in vitro.

Methods: Sterile powder alginate (G/M composition) was mixed with Potassium Phosphate and Sodium Chloride (0.1 M K₂HPO₄ and 0.135 M NaCl, pH 7.4) previously sterilized by autoclave. This solution was then added to sterile powder VEGF-C at three different concentrations 50, 100 and 200 ng/ml. The alginate-VEGF-C mixture was then extruded through a 25g needle and solidified under sterile conditions by internal gelation method/internal setting (Ca²⁺). Previously harvested and cultured Lymphatic Endothelial Cells from rat thoracic duct were placed on a petri dish and cultured (EC media) with the various alginate-VEGF-C hydrogels and a plane alginate hydrogel as control.

Results: The LEC migrated towards all of the alginate-VEGF-C hydrogels and proliferated along their length forming tubular structures, however, the higher concentration of 200 ng/ml of VEGF-C exhibited a more profound effect on migration as well as proliferation. The plane alginate hydrogel had no effect on LEC migration or proliferation and there were no tubular structures formed.

Conclusions: The results of this in vitro study indicate that an alginate biodegradable hydrogel may be an effective delivery system for VEGF-C. Its potent mitogenic effect on LEC could play an important role in Lymphangiogenesis. Further in vivo animal studies are required to evaluate alginate-VEGF-C hydrogels and their potential role in restoring the lymphatic circulation in Lymphedema.

**VEGF SIGNALING IS REQUIRED FOR THE
ASSEMBLY BUT NOT THE MAINTENANCE OF
EMBRYONIC BLOOD VESSELS**

**Christopher J. Drake, W. Scott Argraves,
Amanda C. LaRue, and Paul A. Fleming**

Cardiovascular Developmental Biology Center, Department
of Cell Biology, Medical University of South Carolina,
173 Ashley Avenue, Charleston, SC 29425

drakec@musc.edu

The importance of VEGF signaling to the de novo formation of blood vessels, vasculogenesis and angiogenesis, as compared to the maintenance of blood vessels was analyzed in avian embryos and in a novel in vitro neovascular model, the murine allantois explant culture system. We report that treatment with soluble VEGF receptor 1 (sFlt1) or VEGF antibodies inhibited the de novo formation of blood vessels that occurs in murine 7.5 dpc allantois explant cultures and in 6 somite stage quail embryos. The specific phase of blood vessel formation that was blocked were the processes by which pre-endothelial cells/angioblasts organize to form nascent vessels. In contrast, when vascularized allantois explants (8.5 dpc) or embryos (13 somite stage) were treated with sFlt1 or VEGF antibodies, no discernable alterations to the pre-existing blood vessels were observed. These observations indicate that while VEGF signaling is required for the genesis of new blood vessels, there is no apparent requirement for VEGF signaling to maintain blood vessel morphology. These findings provide insights into the mechanisms by which VEGF antagonists act therapeutically to prevent neovascularization.

EFFECT OF ANTIANGIOGENIC AGENTS ON TUMOR VASCULATURE AND OXYGENATION IN MAMMARY CARCINOMAS

Bruce M. Fenton, Ph.D., and Ivan Ding, M.D.

University of Rochester Medical Center

bruce.fenton@rochester.edu

The effectiveness of therapy in human breast tumors is closely related to alterations in tumor vascular structure and oxygenation. These depend in part on the ability of the tumor to elicit new blood vessel formation (angiogenesis) to supply the rapidly expanding tumor mass. The primary objective of the current studies was to characterize changes in tumor vascular structure, perfusion, and oxygenation as a function of endogenous angiogenic growth factor expression. A second aim was to quantitate such pathophysiological changes following administration of various anti-angiogenic agents. Since such anti-angiogenic approaches will most likely be combined with conventional therapies, it is vital to understand acute and chronic alterations in the tumor micro-environment following specific dosing and scheduling. To investigate the interdependence among tumor growth factor expression and pathophysiology, MCF-7 xenografts were transfected with either fibroblast growth factor-1 (FGF1), FGF4, or three different VEGF isoforms. In addition, two murine mammary carcinomas were selected based on previously documented differences in: 1) VEGF expression, 2) p53 status, 3) metastatic potential, 4) vascularity, and 5) differentiation status. A combination of immunohistochemical stained images of the same frozen tumor sections were obtained to quantify changes in total and perfused vascular spacing and EF5 hypoxia marker intensities in relation to tumor growth in control and treated tumors. Overexpression of FGF or VEGF isoforms produced substantial alterations in tumor vascular configuration, tumor growth, and tumor oxygenation. Pathophysiological response to antiangiogenic therapy varied markedly among the different tumor types and results suggested a direct relationship to endogenous VEGF levels. An intriguing finding was that acute administration of some antiangiogenic agents produced an increase in tumor oxygenation and perfusion, rather than the expected increase in tumor hypoxia. This proposal is clinically relevant in terms of clarifying the relationships among tumor angiogenesis, vascular function, and metastatic potential and provides important clues as to the expected advantages and disadvantages of combining anti-angiogenic approaches with conventional therapies.

**VASCULAR ENDOTHELIAL GROWTH FACTOR
RECEPTOR-1 ACTS AS AN ENDOGENOUS
NEGATIVE REGULATOR OF BLOOD VESSEL
FORMATION**

**Joseph B. Kearney, Carrie Ambler, Kelli-Ann Monaco,
Natalie Johnson, Rebecca G. Rapoport,
and Victoria L. Bautch, Ph.D.**

University of North Carolina-Chapel Hill

xamot@email.unc.edu

Breast cancer progression is dependent upon the ability of breast tumors to recruit new blood vessels by inducing the migration and proliferation of endothelial cells, a process called tumor angiogenesis. Previous studies have established that tumor angiogenesis and the angiogenesis that occurs during mouse embryonic development are similar at the cell biological and molecular levels. Thus, we study embryonic angiogenesis and the genes that affect this process in order to better understand the molecular cues that regulate the process of tumor angiogenesis. Previous studies in the mouse have elucidated the essential nature of VEGF (vascular endothelial growth factor) and its high-affinity receptor tyrosine kinases, flk-1 (VEGFR-2) and flt-1 (VEGFR-1), in the processes of tumor and embryonic angiogenesis. While VEGF and flk-1 have been shown to act as positive regulators of angiogenesis, the role of flt-1 is less well understood. Genetic ablation of the flt-1 receptor does not block angiogenesis during mouse blood vessel development, however, flt-1 null embryos die, presumably from vascular disorganization.

To better characterize the effect of the flt-1 mutation on angiogenesis, we have obtained flt-1 null embryonic stem (ES) cells. ES cells can be induced to undergo differentiation in vitro, where they form a variety of cell types, as well as vascular tissue, which closely resembles the mouse yolk sac vasculature. Differentiation of flt-1 mutant ES cells showed that vascular overgrowth occurs in the absence of flt-1. Determination of endothelial cell mitotic indices in flt-1 mutant ES cultures and well as mouse embryos showed that flt-1 mutants have approximately a 2-fold increase in endothelial cell division. Partial rescue of the flt-1 mutant phenotype was obtained by co-culture of wild-type ES cells with flt-1 mutant ES cells, as well as by treatment of flt-1 mutant cells with a commercially available soluble version of the flt-1 protein. We conclude that flt-1 functions as a negative regulator of angiogenesis by limiting endothelial cell division and that a natural, soluble version of the receptor (sflt-1) partially mediates this effect. These data suggest a potential therapeutic role for flt-1 in negatively modulating tumor angiogenesis.

INTEGRIN-MEDIATED BREAST CANCER CELL ADHESION TO THE ANGIOGENIC FACTOR CYR61

Chih-Chiun Chen and Lester F. Lau

University of Illinois at Chicago, College of Medicine

lflau@uic.edu

CYR61 (cysteine-rich 61, CCN1), a 40 kDa secreted protein associated with the extracellular matrix, has been shown to be a potent angiogenic inducer in vivo. Expression of CYR61 is associated with ~40% of human breast cancer, and up to 70% in cancer of later stages. It is thus of interest to investigate the mechanism by which CYR61 may play a role in breast cancer. Using recombinant baculovirus and insect cell cultures, we have purified recombinant human CYR61 in a biologically active form. Purified CYR61 has multiple biological activities in vitro, including induction of cell adhesion and migration, enhancement of cell proliferation, promotion of cell survival, and regulation of gene expression in endothelial cells and in fibroblasts. Most of these activities have been shown to be mediated through specific cell surface receptors, including integrin alpha v beta 3, alpha v beta 5 and alpha 6 beta 1, as well as the co-receptor heparin sulfate proteoglycans (HSPGs). Indeed, receptor binding experiments showed that CYR61 is the ligand of and binds directly to integrin alpha v beta 3 and alpha v beta 5.

To understand its functional roles in breast cancer, we have addressed the activities of purified CYR61 on mammary adenocarcinoma cells. We show that human mammary adenocarcinoma cells lines, including MCF-7 and T47-D, can adhere to purified CYR61 in serum-free condition within 30 minutes. Further experiments showed that cell adhesion to CYR61 can be inhibited by function-blocking monoclonal antibodies against both integrin alpha 6 and beta 1 subunits, as well as by soluble heparin in the assay media. These data lead us to conclude that human breast cancer cells utilize integrin alpha v beta 3 and cell surface HSPGs to interact with CYR61. We are currently investigating the functional consequence of CYR61 interacting directly with breast cancer cells.

We have constructed an MCF-7-derived cell line that expresses CYR61 under an isopropyl-b-D-thiogalactopyranoside (IPTG)-inducible expression vector. MCF-7 cells express very low endogenous levels of CYR61, making it a suitable model to examine the effects of exogenously expressed CYR61. This expression system will be used to evaluate the role of CYR61 expression in breast cancer development.

ACTIVATED NOTCH4 INHIBITS ANGIOGENESIS: ROLE OF β 1-INTEGRIN ACTIVATION

**Kevin G. Leong, Xiaolong Hu, Linheng Li,
Michela Nosedà, Bruno Larrivee, Christopher Hull,
Leroy Hood, Fred Wong, and Aly Karsan**

Departments of Experimental Medicine, Pathology and
Laboratory Medicine, and Medical Biophysics, University
of British Columbia and British Columbia Cancer Agency,
Vancouver, British Columbia V5Z 1L3, Canada;
Stem Cell Research Laboratory, Stowers Institute for
Medical Research, Kansas City, MO 64110;
and Institute for Systems Biology, Seattle, WA 98105

kleong@bccancer.bc.ca

Notch4 is a member of the Notch family of transmembrane receptors that is expressed primarily on endothelial cells. Activation of Notch in various cell systems has been shown to regulate cell fate decisions. The sprouting of endothelial cells from microvessels, or angiogenesis, involves the modulation of the endothelial cell phenotype. Based on the function of other Notch family members and the expression pattern of Notch4, we postulated that Notch4 activation would modulate angiogenesis. Using an in vitro endothelial-sprouting assay, we show that expression of constitutively active Notch4 in human dermal microvascular endothelial cells (HMEC-1) inhibits endothelial sprouting. We also show that activated Notch4 inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis in the chick chorioallantoic membrane in vivo. Activated Notch4 does not inhibit HMEC-1 proliferation or migration through fibrinogen. However, migration through collagen is inhibited. Our data show that Notch4 cells exhibit increased β 1-integrin-mediated adhesion to collagen. HMEC-1 expressing activated Notch4 do not have increased surface expression of β 1-integrins. Rather, we demonstrate that Notch4-expressing cells display β 1-integrin in an active, high-affinity conformation. Furthermore, using function-activating β 1-integrin antibodies, we demonstrate that activation of β 1-integrins is sufficient to inhibit VEGF-induced endothelial sprouting in vitro and angiogenesis in vivo. Our findings suggest that constitutive Notch4 activation in endothelial cells inhibits angiogenesis in part by promoting β 1-integrin-mediated adhesion to the underlying matrix. The potential anti-angiogenic effects of Notch4 could be exploited as a novel therapy with which to treat breast cancer progression and metastasis.

RESISTANCE TO MAMMARY CARCINOGENESIS IN COPENHAGEN RATS: POTENTIAL ROLES OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND MAST CELLS

Chengshi Quan, Hanlin L. Wang, and Shi-Jiang Lu

Department of Pediatrics and Cancer Center, University of
Illinois at Chicago, Chicago, IL 60612; and Department of
Pathology and Immunology, Washington University School
of Medicine, St. Louis, MO 63110

sjlu@uic.edu

Rats, like human, vary considerably in their susceptibility to the development of mammary cancer among different strains. The Copenhagen rat is extremely resistant to the induction of macroscopically detectable mammary carcinoma by a diverse class of carcinogens including N-nitroso-N-methylurea (NMU). Multiple genetic loci have been linked to the resistant phenotype, but the mechanisms underlying the resistance still remain unknown. Evidence has shown that the acquisition of angiogenic capacity is critical for malignant transformation of a cell. We, therefore, decided to investigate whether administration of angiogenic factor would enhance mammary carcinogenesis in the Copenhagen rat. Vascular endothelial growth factor (VEGF) was administered by embedding VEGF-Elvax film, a sustained releasing formula, under the skin of pubescent female Copenhagen rats two weeks after NMU treatment. Six months after NMU exposure, we found no difference in mammary tumor incidence between VEGF treated animals and controls. Analysis of VEGF expression, however, revealed different expression patterns in mammary epithelial cells of various origins. Mammary epithelial cells from pubescent susceptible Buffalo and resistant Copenhagen rats expressed substantial levels of VEGF messages, whereas cells prepared from 230-day old rats showed negligible levels of VEGF mRNA. We also demonstrated that mammary epithelial cells from tumors developed in susceptible Buffalo rats expressed VEGF mRNA, whereas VEGF messages were barely detectable in mammary epithelial cells prepared from tumors induced in Copenhagen rats. Furthermore, enlargement of the intramammary lymph nodes with prominent mast cells was observed in NMU treated Copenhagen rats with or without VEGF exposure, but not in NMU treated Buffalo rats. Although these data suggest that down regulation of VEGF expression may play a role, but is not sufficient, in conferring resistance to mammary carcinogenesis in the Copenhagen rat, additional mechanisms, likely at the downstream signaling pathway of VEGF, may also occur in resistant strain. Furthermore, enhanced immune response, as evidenced by intramammary lymph node enlargement with mast cell accumulation, may also play a role in conferring resistance in the Copenhagen rat.

FIBRINOLYSIS IN TUMOR-ASSOCIATED ANGIOGENESIS

McLeskey, Sandra W., and Watson, Patricia A.

University of Maryland at Baltimore, Baltimore, MD

mcleskey@son.umaryland.edu

We previously isolated tumor endothelial cells from xenograft tumors and from mammary fat pads of nude mice. RNA obtained from isolated tumor- and mammary fat pad-associated endothelial cells was used to synthesize cDNA which was used for differential cloning techniques in which gene expression in tumor-associated endothelial cells was compared with that of mammary fat pad endothelial cells. Preliminary results show upregulation of expression for uPA, tPA, PAI-1, and MT1-MMP in tumor-associated endothelial cells as compared with mammary fat pad endothelial cells. This upregulation possibly occurs since tumor extracellular matrix is rich in fibrin. Therefore angiogenic endothelial cells may need fibrinolytic capability to invade the tumor matrix. On the other hand, PAI-1 expression has been associated with poor prognosis in breast cancer and evidence indicates that it may be associated with increased motility. This project is intended to elucidate the roles of these fibrinolysis-associated molecules in tumor angiogenesis.

Methods: A mammary fat pad vessel assay is being developed in which the central vessel of the inguinal mammary fat pad is harvested, cut into short segments, and placed in a fibrin matrix. After approximately one week, endothelial cells migrate from the explant into the matrix, forming tube-like structures. We can identify the endothelial cells with immunofluorescence for PECAM-1, an endothelial cell-surface molecule, and with DiI-labeled LDL uptake. We are developing methods to use immunofluorescence to localize molecules of interest to these structures with the hope that invading tips will express fibrinolytic enzymes while more stable structures nearer the explant will not. In addition, we are using antisense technology to abrogate the expression of molecules of interest in the vessel assays.

Results: We are able to identify invading endothelial cells with immunofluorescence. Our results with immunofluorescence for tPA, uPA, PAI-1, and uPAR will be presented.

Development of an in vitro angiogenesis assay specific to breast cancer will enable elucidation of the role fibrinolysis-associated molecules in tumor-associated angiogenesis but also has applicability for other molecules in other matrix materials.

ANTI-ANGIOGENIC STRATEGIES BASED ON TETRATHIOMOLYBDATE-INDUCED COPPER DEFICIENCY

Quintin Pan, George J. Brewer, and Sofia D. Merajver

University of Michigan Health System

smerajve@umich.edu

Copper is a required co-factor for the production and function of angiogenesis mediators. It has been demonstrated in several laboratories that key angiogenesis stimulators function poorly in copper depleted environments. We have hypothesized that moderate copper deficiency is inhibitory of tumor angiogenesis without other major cellular toxicities. In order to investigate the cellular bases for this hypothesis, we have studied the function of human umbilical vein endothelial cells (HUVECs) stimulated by basic fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF) in an environment progressively depleted of copper by chelation with tetrathiomolybdate (TM). TM inhibited FGF2-stimulated tubule formation in HUVECs on Matrigel without affecting VEGF-induced proliferation rates. Copper-depleted extracellular milieu resulted in decreased production of angiogenic chemokines and cytokines into the conditioned media of aggressive breast cancer cells. These cells subsequently grew poorly as orthotopic xenografts in copper depleted nude mice. Over the last few years, we undertook the investigation of TM as an anticancer agent in several pre-clinical models with the aim of understanding in detail the molecular mechanisms behind this apparent global inhibition of angiogenesis. Recent work in our laboratory has shown that TM induced copper deficiency inhibits NFkB mediated transcription of several key angiogenic factors such as IL-6 and IL-8 and a decrease in expression of the p50 and p65 components of the NFkB complex. In agreement with these findings, combinations of TM with chemotherapeutic agents and radiation have proven additive or synergistic in several tumor types, suggesting future applications of this modality to both inhibit the angiogenic switch in minimal disease as well as induce potentiation of apoptosis in conjunction with cytotoxic modalities in the treatment of primary tumors.

CENTRAL ROLE OF P53 ON REGULATION OF VPF/VEGF EXPRESSION IN BREAST CANCER

**Soumitro Pal, Kaustubh Datta,
and Debabrata Mukhopadhyay**

Departments of Pathology, Beth Israel Deaconess Medical
Center and Harvard Medical School, Boston, MA 02215

dmukhopa@caregroup.harvard.edu

The process of angiogenic switching is one of the most important factors in the growth and development of breast tumors. Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is considered to be the most important directly-acting angiogenic protein, that has been shown to be upregulated in breast cancer cells. Hypoxia appears to be an important stimulus for inducing VPF/VEGF mRNA expression in human mammary tumors. Here, we have studied the roles of the tumor suppressor gene p53 and the proto-oncogene c-Src in regulating the transcription of VPF/VEGF in breast cancer cell lines MCF-7 and MDA-MB 435 under both normoxic and hypoxic conditions. p53 significantly inhibited the transcription of VPF/VEGF involving the transcription factor Sp-1. Increased binding of Sp-1 to the VPF/VEGF promoter has been observed, when the cells were exposed to hypoxia. It has been shown that p53 makes a complex with Sp1 and inhibits its binding to the VPF/VEGF promoter to prevent the transcriptional activation. Furthermore, c-Src kinase activity was found to be increased in hypoxic condition and, in the presence of antisense of Src, there was downregulation of the total mRNA level and also the promoter activity of VPF/VEGF. The present study indicates that p53 can also inhibit the hypoxic induction of Src kinase activity and thereby may prevent VPF/VEGF transcription. Taken together, our data suggest a central role of p53, through which it can inhibit VPF/VEGF expression by regulating the transcriptional activity of Sp-1 and also by downregulating the Src kinase activity, under both normoxic and hypoxic conditions.

DISTINCT ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE IN VPF/VEGF-INDUCED ANGIOGENESIS IN MAMMARY CARCINOMA

**Susanta Mondal, Ping Wang, Harold F. Dvorak,
and Debabrata Mukhopadhyay**

Harvard Medical School and Beth Israel Deaconess Medical
Center, Boston, MA 02215

dmukhopa@caregroup.harvard.edu

Angiogenesis, the formation of new blood capillaries from the preexisting vessels, is tightly controlled by the balance of positive and negative regulatory pathways. Molecules that serve as inducers of angiogenesis include fibroblast growth factor, platelet derived growth factor, vascular permeability factor or vascular endothelial growth factor (VPF/VEGF). Angiogenesis measured in primary breast cancer is a major prognostic factor in both node negative and node positive patients, but the molecular mechanisms that control angiogenesis in vivo are not yet well defined. Breast tumors (indeed, all solid tumors) are incapable of growth beyond a certain critical diameter without new vessel formation. Crucial in breast tumors angiogenesis are the paracrine actions of tumor-secreted factors, including VPF/VEGF. Expression of VPF/VEGF mRNA detected by reverse transcriptase-polymerase chain reaction in breast tumors correlates with its tumor-related characteristics of angiogenesis and metastasis potential. Again VPF/VEGF receptors (KDR/FLK-1 and Flt-1) are highly expressed by the endothelial cells in blood vessels of breast tumors. Hence, it is evident that breast tumor produce ample amount of VPF/VEGF, which stimulates the proliferation and migration of endothelial cells, thereby inducing such tumor vascularization by a paracrine mechanism. Overall evidences, as per our knowledge, suggest that production of nitric oxide (NO) is a key component of VPF/VEGF induced vascular hyperpermeability and endothelial nitric oxide synthase (eNOS) plays an important role in this regard. In order to address the question whether eNOS is essential for breast tumor growth and development, we have utilized a transgenic mouse model that is a homozygous knockout of eNOS gene. Therefore, we challenged breast tumor cells in a microenvironment where there will be no endothelial NO synthesis in the presence of VPF/VEGF that is secreted by the tumor cells. We expected that if NO production is the key component for VPF/VEGF induced vascular hyperpermeability, and VPF/VEGF is the sole factor for leakiness of tumor blood vessels, hence the end result would be the halting of tumor stroma formation, thereby reversion of a malignant tumor into a non-malignant phenotype without inhibition of tumor cell proliferation. Surprisingly, from our experimental data it is clearly suggest that BW10232 tumor (a mouse breast tumor cell line) can grow in C57BL/6J eNOS^{-/-} mice and the rate of the tumor growth is even higher as compared to that of control groups. We have also examined the pathways of eNOS activation by VPF/VEGF in vascular endothelium and defined a novel pathway that is initiated by VEGFR-1/ Flt-1 and that channels a negative signaling to VEGFR-2 mediated proliferation. Taken together our data suggest that there might be more diverse role of eNOS in VPF/VEGF-mediated angiogenesis. Our works also initiate better understanding of breast tumor angiogenesis and show the complex nature of VPF/VEGF-mediated signaling in vascular endothelium.

**THE ANGIOGENIC FACTOR FGF-BP1 IS
A NOVEL TARGET GENE OF BETA-CATENIN
IN BREAST CANCER**

**Ray R, Cabal-Manzano R, Byers SW,
Riegel AT, and Wellstein A**

Lombardi Cancer Center, Georgetown University

rayr@georgetown.edu

Studies in our laboratory have shown that the secreted angiogenic factor Fibroblast Growth Factor-Binding Protein 1 (FGF-BP1) is overexpressed in breast cancer. Additionally, our laboratory has shown that FGF-BP1 can act as an angiogenic switch molecule in squamous cell carcinoma and colon cancer, as ribozyme-mediated depletion of FGF-BP1 mRNA in cell lines results in reduced angiogenesis and tumor size of mouse xenograft tumors.

For this study we were interested in establishing an *in vivo* murine model for the study of angiogenesis and FGF-BP1 expression in breast cancer. The *Apc*^{min} mouse is a murine model of the human cancer syndrome- familial adenomatous polyposis (FAP). Over their lifetime, these mice develop intestinal polyps, mammary adenocarcinomas, mammary keratoacanthomas, and desmoid tumors. We have found, by *in situ* hybridization, that expression levels of FGF-BP1 mRNA are significantly elevated in the mammary tumors of the *Apc*^{min} mouse. We also find that β -catenin can up-regulate levels of FGF-BP1, *in vitro*, via increase in FGF-BP1 promoter activity in breast cancer cell lines. Treatment of MDA-MB-468 breast cancer cell line with lithium chloride (LiCl), which stabilizes cytoplasmic β -catenin, resulted in a significant increase in FGF-BP mRNA levels. Furthermore, transient co-transfection assays in the SK-BR-3 breast cancer cell line show that β -catenin can up-regulate FGF-BP1 promoter activity. Thus, we conclude that β -catenin may promote angiogenesis in mammary tumors of the *Apc*^{min} mouse partly through regulation of FGF-BP1.

LONG-ACTING ENDOSTATIN FOR TREATING BREAST CANCER

Mary S. Rosendahl and Stephen P. Eisenberg

Bolder BioTechnology, Inc., 4056 Youngfield,
Wheat Ridge, CO 80033

rrosen100@aol.com

Endostatin is a 20 kDa non-glycosylated protein that is a specific inhibitor of endothelial proliferation and a potent anti-angiogenesis agent. The therapeutic effects of endostatin have been difficult to evaluate for several reasons. Soluble preparations of endostatin quickly lose activity and are plagued by technical difficulties related to storage, handling, and purification methods. In addition, because of its relatively short serum half-life, large quantities of protein are needed to see significant bioactivity in vivo. The goal of our research is to create novel endostatin proteins with both improved physical characteristics and enhanced in vitro and in vivo activities. Our approach towards accomplishing this task involves modifying endostatin by the site-specific covalent addition of a polyethylene glycol (PEG) moiety. By using the known structure of endostatin, we have been able to engineer into the protein, specific cysteine mutations to serve as attachment sites for thiol-reactive PEGs. This strategy allows for the rational design of fully active protein analogues of defined structure and overcomes the problem of loss of bioactivity and product heterogeneity when proteins are modified using standard lysine-reactive PEG reagents.

To date, methods have been developed for the expression, refolding, and purification of the recombinant cysteine endostatin muteins. Our optimized PEGylation efficiencies are greater than 90% for a single site attachment. We are currently evaluating a number of PEGylated endostatin muteins, both in vitro and in vivo. Pharmacokinetic experiments in rats have indicated that, as expected, the PEGylated endostatin proteins have significantly longer half-lives than the parent molecule. We are in the process of selecting a final PEGylated endostatin protein for testing in animal tumor models.

Based upon our experience and literature reports, PEGylated human endostatin should have enhanced stability, greater potency and lower dose requirements. This will allow a larger number of patients access to the drug since it is estimated that more than 9 million cancer patients may benefit from anti-angiogenesis therapy.

MICE EXPRESSING GAIN-OF-FUNCTION FPS/FES ARE CHARACTERIZED BY VASCULAR MALFORMATION, ABERRANT LEVELS OF CIRCULATING BLOOD CELLS, AND POTENTIAL DEFECTS IN ENDOTHELIAL, PLATELET, AND MACROPHAGE FUNCTION

Waheed Sangrar,¹ Jeff Mewburn,² Yotis A. Senis,³ Ralph A. Zirngibl,³
Man Y. Tse,⁴ Sandra G. Vincent,² Michelle L. Scott,³ Steven N. C. Pang,⁴
David H. Lee,⁵ John T. Fisher,² and Peter A. Greer^{1,3}

¹Division of Cancer Biology and Genetics, Queen's University Cancer Research Institute;

²Department of Physiology; ³Department of Pathology; ⁴Department of Anatomy and Cell Biology; ⁵Department of Medicine; Queen's University, Kingston, Ontario, CA K7L 3N6

ws4@post.queensu.ca

Fps is a cytoplasmic tyrosine kinase implicated to have functions in hematopoiesis and angiogenesis and more recently in inflammation and the innate immune system. In order to examine the role of Fps in physiological processes relevant to tumorigenesis, we have engineered a gain-of-function Fps (MFps) mouse model (*Mfps* mice). Remarkably, *Mfps* mice display a complex array of phenotypes. The most striking phenotype was observed using intravital microscopy analyses; this technique revealed highly disorganized and malformed vasculature in cremaster muscle implicating Fps in angiogenesis, a process that is critical to tumor progression. Quantification of vascular area and length per unit area of cremaster muscle tissue showed that these parameters are 1.7-fold higher in *Mfps* mice. These defects were compounded by observations of cardiomegaly, splenomegaly and compromised inflammatory-mediated vascular permeability. In addition, hematological analyses indicated that red blood cell (RBC), reticulocyte and platelet counts are reduced while neutrophil, basophil and monocyte counts are increased in *Mfps* mice. These fluctuations were accompanied with morphological defects in RBCs and platelets including increases in mean cellular volume. Interestingly, alterations in myeloid progenitor populations in the bone marrow correlate with, and may underlie the alterations in output levels of corresponding mature blood cell types. In related studies we employed a mouse model of mammary carcinoma and observed earlier onset of tumors in the context of loss-of-function *fps* genetic backgrounds suggesting that Fps may have tumor suppressor-like properties. The mechanisms that underlie this diverse array of phenotypes are complex and at present, are not clearly understood. However, biochemical studies using immortalized endothelial cell lines derived from *Mfps* mice demonstrated that the presence of MFps in these lines sensitizes these cells to PDGF, a growth factor essential for vasculogenesis. This sensitization may underlie the vascular defects observed in *Mfps* mice. We have also observed defects in aggregation of *Mfps* platelets and increased STAT3/5 signaling in *Mfps* macrophages. The expression and function of Fps in the latter three cell types substantiates a role for Fps in angiogenic, coagulative, immune, and inflammatory function. Given the relevance of these functions to the process of tumorigenesis, it will be important to establish whether Fps exerts its observed tumor suppressor-like properties through regulation of one or more of these functions. Such an understanding will shed light on tumor suppressor mechanisms in general and will provide a rationale for the development of therapeutics designed to mimic normal tumor suppressor function which is compromised in most tumors including breast carcinoma.

Biochemical analysis using viral-mediated ectopic heterogeneous expression of Fps mutants in C166 lines has revealed that MFps, but not Fps is activated in response to PDGF in these cells. This sensitization may underlie the angiogenic phenotype observed in these mice.

Methods: Flow cytometry; peripheral white blood cell (WBC) analysis; western analysis of GM-CSF stimulated bone-marrow (BM) macrophages and Fps expression in immuno-magnetically purified T- and B-cells.

Results: Mice expressing myristylated Fps (Fps^{MF} mice) have normal fertility, but may have slight reductions in viability. Total WBC counts were normal, however there were elevated levels of neutrophils, monocytes and basophils and reduced levels of lymphocytes. Lineage analysis of BM revealed subtle but statistically significant rises in undifferentiated granulocyte and monocyte progenitor populations ($p = 0.015$ and $p = 0.024$ respectively; $n = 22-24$) with proportionate decreases in immature B-cell levels ($p = 0.02$). Elevated CD4+/CD8- populations were present in the thymus (1.6-fold) while reduced levels were observed in the spleen (0.38-fold). Interestingly, Fps, Fps^{MF} and Fer were found to be highly expressed in thymocytes and mature T-cells purified from spleen. Analysis of GM-CSF signaling in Fps^{MF} BM macrophages displayed subtle alterations in the kinetics of activation of the GM-CSF receptor β -chain. Enhanced STAT3/5 was also observed, while Jak2 phosphorylation profiles were unaffected.

Conclusions: Potentiation of undifferentiated myeloid progenitors and immature B-cells by Fps^{MF} may underlie the observed increases in granulocyte and monocyte output and the corresponding decreases in lymphocyte output. These data suggest that Fps^{MF} may promote myeloid differentiation pathways. This possibility is substantiated by enhanced STAT3 and STAT5 phosphorylation, both of which are heavily implicated in myeloid differentiation and survival. Interestingly, Jak2 phosphorylation was unaltered suggesting that Fps^{MF} alters STAT3/5 signaling independently of this kinase. Lastly, observations of aberrant thymocyte maturation in Fps^{MF} mice coupled with observations of Fps and Fps^{MF} expression in mature T-cells suggests that Fps may play a role in T-cell signaling and/or selection.

ANTAGONISM OF BREAST TUMOR ANGIOGENESIS

**Donald R. Senger, Mien Van Hoang,
and Mary C. Whelan**

Department of Pathology
Beth Israel Deaconess Medical Center

dsenger@caregroup.harvard.edu

The purposes of this study are to identify new strategies for blocking the growth of new blood vessels associated with primary breast tumors and breast tumors metastatic to lung. The development of new tumor blood vessels, a complex process known as tumor angiogenesis or neovascularization, promotes tumor growth; and this process also likely contributes to tumor invasion and metastasis. Consequently, identification of new strategies for suppressing breast tumor angiogenesis may lead to development of new therapies for controlling the growth and spread of breast carcinomas.

To identify new strategies for suppressing breast tumor angiogenesis, we have focused on mechanisms through which collagens and their receptors regulate assembly of endothelial cells (ECs) into mature blood vessels. We determined that the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ cell surface integrins critically mediate interactions between interstitial collagen and human lung microvascular ECs and that antagonism of these two integrins inhibits lung tumor angiogenesis in mice. In marked contrast, we found that neither of these integrins is important for mediating interactions between collagen and human breast microvascular ECs.

Despite differences among breast and lung ECs regarding the relative significance of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ for mediating interactions with interstitial collagen, we found important mechanistic parallels between breast and lung ECs regarding “angiogenesis in vitro”, a dynamic process provoked by three dimensional collagen matrices that imitates important morphogenetic aspects of neovascularization in vivo. In particular, we identified key signaling molecules that regulate proper EC organization in collagen; and with a retroviral strategy, we are testing the significance of these molecules for breast carcinoma angiogenesis in mouse mammary fat pad. We expect these studies to identify new molecular targets for suppressing breast tumor angiogenesis and thereby identify new rational strategies for controlling breast tumor growth.

ANTIANGIOGENIC ACTION OF CHEMICALLY MODIFIED TETRACYCLINES IN BREAST CANCER

Sanford R. Simon and Mansi Kothari

Departments of Biochemistry and Pathology, State
University of New York at Stony Brook, NY 11794-8691

ssimon@notes.cc.sunysb.edu

Control of breast cancer may be achieved by a combination of interventions, including downregulation of the angiogenic response which maintains tumor growth and proliferation. Proangiogenic signals may emanate from the tumor cells themselves as well as from inflammatory cells which infiltrate the tumors. A nonantimicrobial chemically modified tetracycline, 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-300) is currently being evaluated at the National Cancer Institute in Phase I trials on patients with a variety of solid tumors, and has been shown to reduce the angioproliferative response in Kaposi's Sarcoma. The 9-dimethylamino derivative of CMT-300 (CMT-308), induced less photosensitivity in vitro than the parent compound. Accordingly, we have studied the effects of CMT-300 and CMT-308 on two human breast tumor cell lines with different degrees of invasive and metastatic potential as well as a human monocytoid cell line which serves as a model of tumor-infiltrating macrophages. In the first year of research sponsored by this award, we have studied release of Vascular Endothelial Growth Factor (VEGF) by two breast tumor cell lines, MCF-7 (which retains estrogen responsiveness and is not highly invasive) and MDA-MB-231 (which is estrogen insensitive and is highly invasive), using an Enzyme-Linked Immunosorbant Assay (ELISA) for quantitation. Both lines release VEGF at levels which can be augmented in a dose-dependent fashion by Transforming Growth Factor- β (TGF- β). Consistent with the idea that MDA-MB-231 is a model of late stage aggressive breast cancer and MCF-7 is a model of earlier stage cancer, the former line releases higher levels of VEGF than the latter. The levels of VEGF from both lines are diminished when 20 μ M CMT-308 (a noncytotoxic dose which is comparable to levels of CMT-300 reached in patients in the NCI Phase I trials) is present during culture. This diminution is especially marked in the presence of TGF- β , suggesting that CMT-308 may be affecting a signal transduction pathway which is activated in these tumor cell lines by TGF- β . Levels of VEGF released by both breast tumor lines are diminished much more by CMT-308 than by CMT-300. Because highly vascularized tumors are often also infiltrated with inflammatory cells, we examined the effects of the CMTs on VEGF production by a highly differentiated monocytoid cell line, Mono Mac 6. This cell line releases high levels of VEGF which are not affected by TGF- β , but 20 μ M CMT-308 effectively abrogates all VEGF release. The results on VEGF release indicate that the CMTs may have utility in management of breast cancer by diminishing the pro-angiogenic signals released by the tumors and by infiltrating mononuclear cells. Because the CMTs have been found to be safe and well tolerated in cancer patients as well as normal volunteers, they have promise for rapid development as components of comprehensive therapeutic strategies for breast cancer management.

CHARACTERIZATION OF ANGIOGENESIS IN THE CARCINOGEN-ENU-INDUCED BENIGN AND MALIGNANT MAMMARY TUMOR MODEL

**Min-Ying Su, Ph.D., Hon Yu, M.S.,
Jun Wang, M.D., and Orhan Nalcioglu, Ph.D.**

Center for Functional Onco-Imaging,
University of California, Irvine, CA 92697-5020

msu@uci.edu

As the potential of anti-angiogenic or anti-vascular therapy for cancer treatment becomes more promising, the development of valid animal models as well as therapeutic efficacy markers have become increasingly important. The carcinogen ENU can induce benign and malignant tumors in rats, and the malignant tumors came with various SBR (Scarff-Bloom-Richardson) grades, thus it can be used a suitable model for studies of differential diagnosis as well as malignant tumor grade staging. Assessment of angiogenesis by immunohistochemical (IHC) staining is the most commonly used technique. For each specimen we measured the expression level of mutant p53, thrombospondin-1, Vascular Endothelial Growth Factor (VEGF), and microvessel density using Factor VIII staining. On the other hand, imaging may be complementary to the IHC studies to measure the vascularity. In this study we characterized the angiogenesis status of the ENU induced mammary tumor model using Magnetic Resonance Imaging (MRI) and IHC molecular markers.

N-ethyl-N-nitrosourea (ENU, 90 mg/kg) was injected i.p. into 30-day old SPF Sprague-Dawley rats (n=50) to induce mammary tumors. The tumors started to appear 2 months after injection. The baseline MRI study was performed when the tumor reached approximately 1.0 cm in diameter. For each tumor the enhancement kinetics of two contrast agents, the small agent Gd-DTPA and a mid-sized agent Gadomer-17, were measured. The volumetric growth rates, as well as the early (30-sec) and maximum (approximately 2-min) enhancements between benign and malignant tumors were compared. Each tumor was then surgically removed for IHC staining analysis. The rats were kept for observation of recurrence, further development of other tumors, and metastasis into other organs, to explore whether this model can be used to study recurrence and metastasis as in human breast cancer.

Ninety three tumors were found in 1 year after the injection of ENU. Most tumors belonged to four major types, 2 malignant and 2 benign. The malignant tumors included ductal adenocarcinoma (n=25) and papillary adenocarcinoma (n=21), and the two major benign lesions were fibroadenoma (n=24) and adenosis (n=13). Multiple tumors (up to 5) could develop in one rat, all at different locations. Interestingly no tumor recurrence was observed at the surgical site after removal of a previous tumor. Also, none of the abdominal tumors were from breast origin. We also investigated lymph nodes from some rats, and found no sign of any cancer. In MRI studies, the two malignant tumors exhibited similar enhancement kinetics, showing rapid early enhancing slope and high enhancement magnitude. The two benign lesions had much slower early enhancing slope (highly significantly). In IHC studies, the ductal adenocarcinoma had the highest microvessel density, then in order was papillary adenocarcinoma, fibroadenoma, and the adenosis had the lowest microvessel density. All tumors had wild type p53. VEGF and TSP-1 were not significantly different among the 4 types. Higher vessel density was associated with higher MRI enhancement.

Our results demonstrated that all carcinogen ENU induced tumors were primary tumors, and they did not metastasize to lymph nodes or to other organs. A better characterization of this model may aid in future development of diagnostic or therapeutic agents tested on this model.

INHIBITION OF ENDOTHELIAL CELL SURFACE EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR-2 USING AN INTRABODY STRATEGY

**Yurong Y. Wheeler, Timothy E. Kute,
Mark C. Willingham, Si-Yi Chen, and David C. Sane**

Wake Forest University School of Medicine, Medical
Center Boulevard, Winston-Salem, NC 27157

yyang@wfubmc.edu

Angiogenesis plays a pivotal role in tumor growth and metastasis. VEGF, an endothelial specific mitogen and an angiogenesis inducer in vivo, is one of the most important tumor angiogenesis factors. KDR, a VEGF receptor, appears to be the major transducer of VEGF signals in endothelial cells. A tethered intracellular antibody (“intrabody”) strategy has been used successfully for both phenotypic and functional knockouts of target molecules. In this study, we have extended this intrabody strategy to target the KDR single chain antibody (scFv) p3S5 to the endoplasmic reticulum (ER) to block the surface expression of KDR on endothelial cells. We hypothesize that the KDR intrabody targeted to the lumen of the ER would bind newly synthesized KDR and block their transport to the surface of endothelial cells, thereby inhibiting endothelial cell proliferation and tumor angiogenesis. Various KDR intrabody expression vectors have been generated and transfected into human umbilical vein endothelial cells (HUVECs). The localization and production of the intrabody have been demonstrated by immunofluorescent staining and western blot. Decreased level of KDR surface expression (87.6% of intrabody transfected vs. 33.8% of control vector transfected HUVECs had no KDR expression) has been illustrated by flow cytometry assay. [3H]-thymidine incorporation in intrabody transfected HUVECs has been decreased by 72.5%. Furthermore, an adenovirus expressing this intrabody gene has been generated. More than 90% of HUVECs can be infected with this adenovirus and intrabody gene expression has been demonstrated by western blot. Migration assay will be done using this adenovirus-infected HUVECs to illustrate that endothelial cell migration can be inhibited after expressing this intrabody. The results of this study demonstrate the therapeutic potential of anti-angiogenesis using an intrabody strategy in breast cancer and other solid tumor gene therapy.

**ERBB2 OVEREXPRESSION CORRELATES WITH INCREASED
EXPRESSION OF VEGF-A, VEGF-C, AND VEGF-D IN HUMAN
BREAST CARCINOMA, AND ERBB2-MEDIATED
ANGIOGENESIS IS REDUCED UPON COMBINED TREATMENT
WITH HERCEPTIN AND TAXOL**

**Kristine S. Klos,¹ Wentao Yang,¹ Lianglin Zhang,¹
Xiaoyan Zhou, Sangkyou Lee, and Dihua Yu**

The University of Texas M.D. Anderson Cancer Center
Department of Surgical Oncology

kklos@mail.mdanderson.org - dyu@notes.mdacc.tmc.edu

The erbB2 (or HER2, neu) gene-encoded receptor tyrosine kinase (RTK) belongs to the EGF receptor family. ErbB2 overexpression correlates with poor prognosis and the number of lymph node metastases in breast cancer patients. ErbB2 has been shown to upregulate vascular endothelial growth factor (VEGF) – a potent angiogenic agent that increases vascular permeability and endothelial cell proliferation, migration, and differentiation. Two other members of the VEGF family, VEGF-C and VEGF-D, are angiogenic and potential lymphangiogenic growth factors, however, their relationship with clinicopathologic parameters is not clearly known. Therefore, we looked at the expression levels of VEGF-A, VEGF-C, VEGF-D, and ErbB2 in 107 breast carcinoma cases and 22 benign breast tissues by IHC and quantitated them by image analysis. Higher expression of VEGF-C and VEGF-D was found in breast carcinomas than in benign breast tissues. Importantly, VEGF-A, VEGF-C, and VEGF-D expression was all found to be significantly and positively correlated with ErbB2 expression. High levels of VEGF-A expression were associated with shorter disease-free survival (DFS). Patients with tumors expressing high levels of VEGF-C and VEGF-D showed a notable trend for worse DFS, however, it was not statistically significant. The combination of VEGF-A and VEGF-C status predicted survival better than either marker alone.

The recombinant humanized anti-ErbB2/HER2 monoclonal antibody HerceptinTM (Trastuzumab) has been shown to significantly enhance the tumoricidal effects of anti-tumor drugs such as paclitaxel (taxol) in patients with ErbB2-overexpressing tumors. Since, ErbB2 is correlated with an increase in angiogenic growth factors; we wanted to see if we could decrease ErbB2-mediated angiogenic responses with combined treatments of taxol and Herceptin. MDA-MB-435 human breast cancer cells that express very low levels of the ErbB2 protein were stably transfected with the erbB2 gene to establish ErbB2 over-expressing cells (eB1). The eB1 cells were treated with Herceptin, taxol, or Herceptin plus taxol. In vitro, we see less VEGF-A secretion, reduced endothelial cell migration, and reduced endothelial cell differentiation when eB1 cells are treated with the Herceptin/taxol combination over either treatment alone. SCID mice were injected with the ErbB2-overexpressing cells in a spontaneous metastasis assay. Metastases to the lungs were counted four months after injection. 75% of the mice injected with the eB1 cells suffered from metastases to the lungs. Metastasis dropped to 63% when mice were treated with Herceptin alone, to 57% when mice were treated with taxol alone, and to 44% when mice were treated with both Herceptin and taxol, which correlated with reduced angiogenic activity. This data suggests that combined Herceptin and taxol treatments may have increased tumoricidal effects by reducing ErbB2-mediated angiogenesis.

¹These authors contributed equally to this work.

EFFECTS OF TAMOXIFEN AND GW5638 ON MICROVESSELS IN TUMOR AND UTERINE TISSUES

Fan Yuan,¹ Sheng Tong,¹ Qing Chen,¹
Donald P. McDonnell,² and Mark W. Dewhirst³

Departments of ¹Biomedical Engineering, ²Pharmacology
and Cancer Biology, and ³Radiation Oncology, Duke
University, Durham, NC 27708

fyuan@acpub.duke.edu

Although tamoxifen has shown promising results in treatment and prevention of breast cancer, specific concerns have been raised about its side effects in patients. Therefore, significant efforts have been made to identify new antiestrogenic agents that can replace tamoxifen. GW5638 is a novel non-steroidal antagonist of estrogen receptor (ER). It inhibits the agonist activity of estrogen, tamoxifen, and raloxifene *in vitro*, causes minimal uterotrophic activity in ovariectomized rats, and can inhibit the growth of an ER-positive, tamoxifen-resistant breast tumor in mice. In addition, GW5638 functions as a full ER agonist in bone and the cardiovascular system. On the other hand, previous studies have shown that tamoxifen can affect angiogenesis via either ER-dependent or independent pathways. It may stimulate expression of VEGF which is a potent factor for both angiogenesis and microvascular leakiness. Effects of GW5638 on microvessels are still unknown. Therefore, the goal of our study is to investigate effects of tamoxifen and GW5638 on microvessels in tumor and uterine tissues.

The investigation was based on three animal models: the mouse dorsal skinfold chamber, a mouse fibrin chamber model, and the rat cornea pocket assay. The animals were treated with systemic injections of tamoxifen or GW5638. The effects of these compounds on microvascular density and permeability of albumin as well as tumor size were quantified at the end of treatment. In addition, we investigated the effects of tamoxifen on the tissue morphology and the vascular leakiness in the uterus of mice.

We found that both tamoxifen and GW5638 could reduce bFGF-induced angiogenesis in the rat cornea and had a tendency to decrease the microvascular permeability of albumin in MCF7 tumors. There was no significant difference between the two compounds with respect to these effects, but GW5638 could inhibit the growth of a tamoxifen-dependent tumor. In the uterus, tamoxifen had a significant effect on the morphology of uterine tissues and the microvascular permeability of dextran (molecular weight=70,000) in CD1 mice. However, these effects of tamoxifen were insignificant in nude mice, presumably due to the difference in plasma concentrations of estrogen.

We conclude that both tamoxifen and GW5638 are weak antiangiogenic agents. They may inhibit tumor growth through both direct effects on tumor cells and indirect effects on angiogenesis.

**ACQUIRED EXPRESSION OF A MESENCHYMAL-
SPECIFIC GENE PERIOSTIN BY EPITHELIAL-
DERIVED BREAST CANCERS PROMOTES TUMOR
ANGIOGENESIS**

**Rong Shao, Shideng Bao, Xuefang Bai,
Carrie Blanchette, Ryan M. Anderson,
Jeffrey R. Marks, and Xiao-Fan Wang**

Department of Pharmacology and Cancer Biology,
Department of Experimental Surgery, Duke University
Medical Center, Durham, NC 27710

wang0011@mc.duke.edu

Late stages of tumorigenesis are poorly understood complex processes associated with the expression of genes by cancer cells that promote specific tumorigenic activities, such as angiogenesis and metastasis. Here, we describe the identification by a functional genomics approach of periostin as a mesenchymal-specific gene whose acquired expression by epithelial cell-derived human cancers leads to a significant enhancement in tumor progression and angiogenesis. Undetectable in normal human breast tissues, periostin was found to be overexpressed by a vast majority of human primary breast cancers examined by genearray analysis. This result was confirmed by western blot and immunohistochemical analysis on selected tumor tissue samples derived from human patients. To explore the mechanism by which periostin overexpression may be associated with breast tumorigenesis, we used a model system in which tumor cell lines engineered to overexpress periostin were injected into immune-compromised animals to grow as xenografts. We found that those cells display a phenotype of significantly accelerated growth with the appearance of elevated levels of hemorrhage. Subsequently we found that there is a higher level of blood content and more vascular endothelial cells in periostin-overexpressing tumors than controls. To explore further the mechanism by which the expression of periostin is associated with angiogenesis, we used both recombinant periostin and a co-culture system to evaluate the effects of periostin on the function and activities of human microvessel endothelial cells (HMVEC). We found that the migration and tube-formation abilities of HMVEC were significantly enhanced by the presence of periostin. Finally, the underlying mechanism of periostin-mediated induction of angiogenesis was found to be in part a result of the up-regulation of VEGF receptor Flk-1/KDR. Our findings provide a novel insight into the mechanism by which tumor angiogenesis is promoted through the acquired expression of a mesenchymal-specific gene and implicate the process of epithelial-mesenchymal transformation as a crucial step intimately associated with late stages of tumorigenesis.